EFFECT OF pH AND TEMPERATURE ON JUMBO SQUID PROTEINS

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ABSTRACT

Evaluation of the effect of pH (2 to 13) and temperature (0 to 50C) on functional properties of jumbo squid proteins was performed, followed by a 2 × 3 factorial design for producing squid protein hydrolysates bearing useful functional properties. In particular, the effects of pH (8, 9 and 10) and temperature (30, 35, 40C) were evaluated. Alcalase and papain were tested on each treatment. The protein recovery, whippability and emulsifying capacity of the hydrolysates were evaluated. Almost 80% of the proteins were recovered in water-soluble form after hydrolysis with papain at pH 10 at the three temperatures. The highest values of whippability (245 ± 17.7%), foam stability (100%), emulsion-forming capacity (27 ± 0.97%) and stability (99.99 ± 8.8%) occurred with papain-produced hydrolysates. When squid protein was treated at 50C and pH 8, the highest whippability value (390.0 ± 0.1%) and foam stability (100%) were obtained when no enzyme was added.

PRACTICAL APPLICATIONS

This paper assesses how process variables, particularly temperature and pH, affect the functional properties of squid proteins. Making use of such process variables will produce more useful and efficient processes, as the application of a hydrolysis system. The processing of jumbo squid protein to obtain proteins bearing adequate functional properties would be an inexpensive way to provide added value to this marine resource and the production of a high-quality protein ingredient. These results hold promise for jumbo squid proteins as useful food ingredient because of their functional properties.

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INTRODUCTION

In Mexico, jumbo squid (*Dosidicus gigas*) has low economic value, large capture volumes and largely sold as an unprocessed product, mainly as raw product to Asian countries (SEMARNAP 2000). Yet, squid muscle is a source of high-quality protein because it is readily digestible and possesses all essential amino acids for humans. Appropriate processing of jumbo squid muscle would be a suitable way to obtain enriched products. Recently, a squid surimi production facility opened in the port town of Santa Rosalía in the State of Baja California Sur of Mexico, but it is still a very small operation, compared with the volume of harvested squid.

The failure of some attempts to process jumbo squid is related to the dearth of knowledge of its physiological and intrinsic characteristics. Use and processing of jumbo squid muscle has been limited to feed for aquaculture, in part because of the distinctive acid-bitter flavor of its muscle and also by its "high endogenous proteolytic activity that has poor functional properties" (Gómez-Guillén *et al.* 2003). However, if high proteolytic activity is true, it may be advantageous because autohydrolysis of its own proteins can lead to water-soluble products that can be recovered and used as food ingredients. Squid autohydrolysis was reported for *Illex illecebrosus* and *Loligo pealei*, and the soluble protein was used as an attractive feed ingredient for shrimp (Lian *et al.* 2005). Therefore, autohydrolysis could be a suitable process for recovery of squid protein.

A typical, well-known process used for recovering proteins is enzymatic hydrolysis by adding pure or crude extracts of enzymes. This is a biochemical process that improves some functional properties of proteins, mainly solubility (Adler-Nissen 1986; Gildberg 1993). Processes using hydrolysis are facing the challenge of optimization of improving the downstream steps and taking advantage of inexpensive sources, such as by-catches or fishery residues (Martin 1998). Commercial enzymes are preferred because their action is controllable; autohydrolysis depends on a mix of muscle with enzymes that varies in content and activity.

To propose suitable processes for using jumbo squid, we must determine how the functional properties of proteins are affected by process variables, such as pH and temperature. The addition of an acid or alkali may improve emulsifying properties, which is related to an increase in surface hydrophobicity and surface-interface activity (Kristinsson and Hultin 2003). Increased temperature and heating time also increases surface hydrophobicity and formation of disulfide bridges, which enhances emulsifying properties (Hammershøj *et al.* 2006). However, at high temperatures, increased hydrophobicity leads to denaturation-aggregation, which causes loss of functional properties, mainly solubility (Poulter *et al.* 1985; Tornberg 2005). To resolve these issues

in jumbo squid, this work evaluates the effect of pH and temperature on the functional properties of squid proteins.

MATERIALS AND METHODS

Squid Samples

Jumbo squid were collected offshore at Santa Rosalía, Baja California Sur, Mexico and immediately killed in iced water on the fishing vessel. Mantle length averaged 52.1 cm. Upon landing, the mantle was separated, eviscerated, washed with freshwater, wrapped in plastic bags and kept between ice beds until reaching the laboratory within 16 h after capture. On arrival, mantle portions were stored at −30C until used.

Preparation of Samples

A homogeneous batter was prepared from a pool of several mantles that had been skinned and had the fins removed. Small pieces from each mantle were cut and ground for 30 s at high speed in a kitchen blender. During preparation of the samples, temperature was kept at 2–4C. By adding distilled water or salt solutions, this batter was used to prepare squid homogenates, as described in the further discussion.

Effect of Temperature

Samples of squid homogenate were prepared by adding distilled water to achieve 3% protein content, based on total protein quantification by microkjeldahl (AOAC International 1995). This homogenate was incubated and continuously stirred at constant pH of 5.9 (natural pH) and several temperatures: 0, 10, 20, 30, 40 or 50C. After 1 h, samples were evaluated for solubility, emulsion capacity, emulsion stability, whippability and foam stability. Additionally, as the squid homogenate was prepared, samples were taken to evaluate its functional properties. These latter samples will be called "raw samples" in later discussion.

Effect of pH

Squid homogenate containing 3% protein, based on total protein quantification by the microkjeldahl method (AOAC International 1995) was prepared, as described previously. The resulting homogenate was divided into 12 portions, and the pH of each treatment was adjusted to values from 2–13, using 1 N NaOH or 1 N HCl. All treatments were incubated at 3C with continuous

stirring. After 60 min, aliquots of each treatment were evaluated for solubility of protein at three values of ionic strength (0.05, 0.3 and 0.5, adjusted by adding NaCl), emulsion-forming capacity, emulsion stability, whippability and foam stability. The effect of pH on protein composition on the treated samples was analyzed by SDS-PAGE and compared with a control sample at the natural pH (5.9) and without incubating with continuous stirring.

Enzymatic Hydrolysis

Squid homogenates containing 3% protein, based on total protein quantification by microkjeldahl (AOAC International 1995), were prepared as described previously. A 90-mL sample was placed in a pH Stat system (718 Stat Tritrino, Metrohm, Switzerland) under controlled temperature with continuous stirring. The experimental variables were pH 8, 9 and 10 at 30, 35 and 40C. The combined effect of both factors was evaluated in a 2×3 factorial design, along with the qualitative variable, which was the type of commercial enzyme used to obtain the hydrolysates: alcalase (donated by Novozymes, Salem, Vancouver, Canada) or papain (P4762, Sigma, St. Louis, MO). To solubilize squid proteins and upgrade their functional properties, a low enzyme-substrate (E-S) rate was selected, set as 0.3 and 3 activity units per gram of protein (AU/g) of Alcalase® and papain, respectively, which were determined in previous experiments (not shown). One AU/g is the change in absorbance at 366 nm $\times$ min^{-1} mg^{-1} protein, using azocasein as the substrate, as described in Navarrete del Toro and García-Carreño (2002). After 60 min, samples were cooled on ice to stop hydrolysis and soluble proteins and functional properties (emulsion forming capacity, emulsion stability, whippability and foam stability) of the hydrolysates were evaluated. The degree of hydrolysis (% DH) was determined according to Adler-Nissen (1986).

SDS-PAGE

Squid protein samples from the different treatments were analyzed by SDS-PAGE (Laemmli 1970). Samples containing 50 µg protein taken before and after treatment were mixed with loading buffer: 0.125 M Tris-HCl, 4% SDS, 20%, v/v glycerol, 0.2 M DTT and 0.02% bromophenol blue at pH 6.8, and heated for 5 min in a boiling water bath. Samples were analyzed in a vertical electrophoresis unit (Hoefer, model SE 260, San Francisco, CA), using a 10% polyacrylamide gel for separating protein bands. Electrophoresis was conducted at a constant current (13 mA per gel). The separated protein bands were stained with a solution containing 40% methanol, 7% acetic acid, and 0.5% Coomassie Brilliant Blue R-250. Two molecular mass calibration kits were used (Kit 17-0615-01 for 14 to 97 kDa and Kit 17-0446-01 for 53 to

220 kDa; GE Healthcare Bio-Sciences Corp., Piscataway, NJ). After 2 h, the excess stain was removed with a solution containing 40% methanol and 7% acetic acid.

Functional Properties of Squid Proteins

Protein solubility was determined by dispersing a sample of squid batter in NaCl solutions of 0.05, 0.3 or 0.5 ionic strengths to a final protein concentration of 1.0% measured by the microkjeldahl method. After thorough mixing for 3 min and mild mixing for 30 min at room temperature (25C), the mixture was centrifuged at 12,000 × g at 4C for 20 min. Solubility of the protein content in the supernatant was measured (Lowry *et al.* 1951). Solubility was expressed as a percentage of soluble protein with respect to the total protein in the sample.

Emulsifying capacity was determined by slight modification of the method described in Swift *et al.* (1961), in Hill (1996). Briefly, squid batter was mixed with distilled water to a final protein concentration of 0.3%, to 50 mL of protein suspension, 9 mL sunflower seed oil was added. The mixture was homogenized for 30 s at high speed at room temperature with a propeller impeller and, immediately afterwards, centrifuged at 2,000 × g at 20C for 5 min. The volume of the emulsion was measured for each sample. Emulsifying capacity was calculated as the ratio of the volume from the emulsion formed and the initial mixture.

Emulsion stability of each sample was determined by heating the emulsion to 80C for 30 min. The emulsion volume was measured after heating. The emulsion stability is expressed as a percentage of the remaining emulsifying capacity after the heating period.

Whippability and *foam stability* were measured using the Rudin method (Wilde and Clark 1996) with a slight modification. Briefly, a 40-mL mixture of distilled water and squid batter, with a final protein content of 3%, was homogenized with a flat impeller (almost parallel to the bottom) at high speed for 1 min. The resulting foamy liquid was poured into a graduated cylinder and the foam and liquid volumes were recorded. Whippability was expressed as a percentage of increased volume. Foaming stability was expressed as the volume of foam remaining after allowing the sample to rest at room temperature for 60 min.

Statistical Analysis

All tests were conducted in triplicate. The data reported is expressed as mean values ± SD. One-way analysis of variance, coupled with Tukey's Honestly Significant Difference (HSD) procedure, was used for all tests to determine significant differences among treatments with $P < 0.05$. Statgraphics Plus for Windows® v. 5.0 software performed these operations.

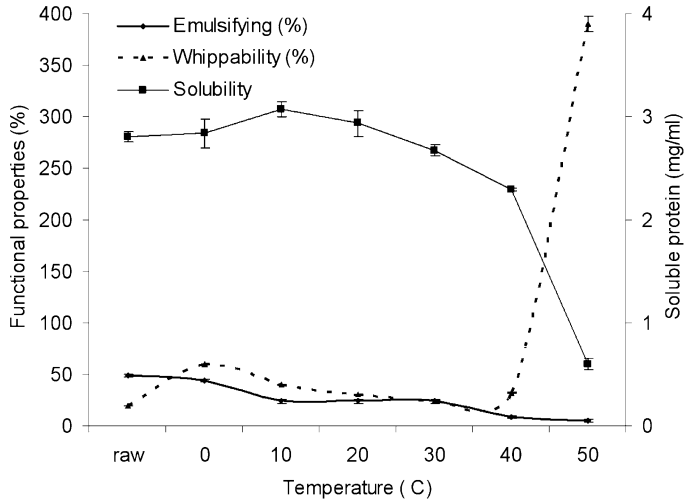

FIG. 1. EFFECT OF TEMPERATURE ON THE FUNCTIONAL PROPERTIES OF JUMBO SQUID PROTEINS
Values represent the mean of at least three replicates; bars indicate SD.

RESULTS AND DISCUSSION

Effect of Temperature

The values obtained for each functional property affected by temperature are shown in Fig. 1. As affected by temperature, functional properties can be divided into three parts. At time zero, when the squid homogenate is prepared, tissues had just been disrupted, and proteins are nearly in their native condition. After incubation and stirring for 60 min at temperatures from 0–30C, interactions occurred between the water in the media and the proteins, leading to decreased solubility and whippability. Emulsion-forming capacity decreased in the range of 0–10C. Stability decreased at 10–30C. Exposure of jumbo squid to temperatures above 40C led to a dramatic loss of solubility and emulsion-forming capacity. Contrary to the reduction in solubility and emulsifying capacity, whippability increased to 390% at 50C. Although some authors reported that increased hydrophobicity enhanced emulsifying capacity (Kristinsson and Hultin 2003; Hammershøj *et al.* 2006), our results show a loss of solubility and emulsion-forming capacity, suggesting that hydrophobic interactions between proteins were favored and resulted in protein aggregations. Similar results were reported for several kinds of muscle proteins, in which myofibrillar proteins aggregated between 40–60C. For these proteins,

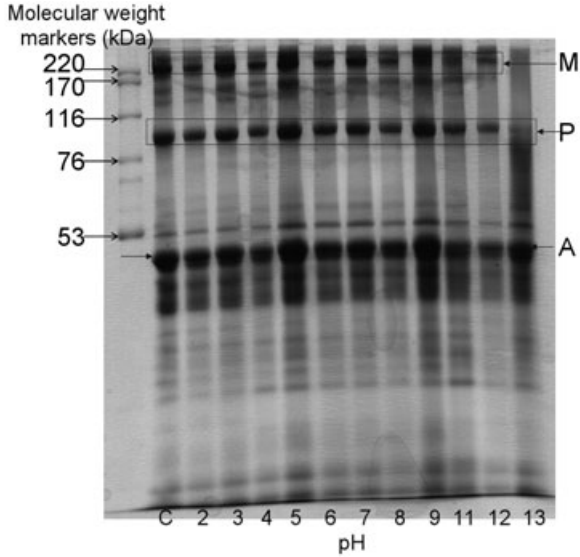

FIG. 2. EFFECT OF pH ON THE PROFILE OF JUMBO SQUID PROTEIN
First lane represents the molecular weight markers (kDa); numbers below lanes indicate the tested pH levels. Myosin (M), actin (A) and paramyosin (P) are indicated. "C" represents the initial pH condition (without change in pH).

unfolding begins at 30–32C, followed by protein association at 45–50C (Kristinsson and Rasco 2000; Tornberg 2005).

Effect of pH

The protein SDS-PAGE profile, after incubation at pH 2–13, is shown in Fig. 2. Proteins, mainly myosin (220 kDa), paramyosin (~111 kDa) and actin (~45 kDa), were affected after incubation at pH 13. The functional properties of solubility, emulsion-forming capacity and whippability undergo changes in different ways along the pH scale (Fig. 3–5).

We observed that an increase in ionic strength (μ) reduced the global isoelectric point (pI) and the lowest solubility of squid proteins occurred at pH 5 at $\mu = 0.05$. At $\mu = 0.3$, the global pI was at pH 4, and at $\mu = 0.5$, the global pI was at pH 3 (Fig. 3). At pH 12 and 13, the highest solubility was achieved at $\mu = 0.5$, whereas at pH 2 to 4, the highest solubility was reached at $\mu = 0.05$. How the solubility of proteins is affected by pH and μ is important in food technology, since they are the main variables in protein extraction procedures (Hashimoto *et al.* 1979; Kristinsson and Hultin 2003). A protein treatment using both methods, change in pH and μ, could be a suitable option to improve its functional properties (Sánchez-Alonso *et al.* 2007).

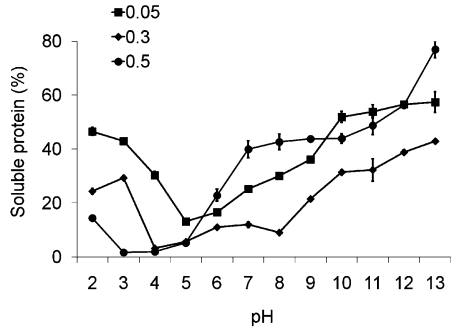

FIG. 3. EFFECT OF pH ON THE SOLUBILITY OF JUMBO SQUID PROTEIN AT DIFFERENT IONIC STRENGTHS
Different bullets indicate levels of ionic strength tested. Values represent the mean of at least three replicates; bars indicate SD.

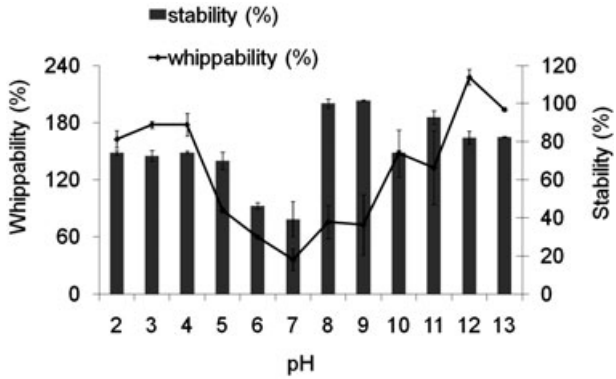

FIG. 4. EFFECT OF pH ON THE WHIPPABILITY AND FOAM STABILITY OF JUMBO SQUID PROTEINS
Values represent the mean of at least three replicates; bars indicate SD.

Along the pH scale, whippability had the lowest value (<60%) at pH 7. The highest whippability occurred at pH 12 (Fig. 4). However, the highest stability, 100% at pH 8 to 9, cannot be disregarded, because it appears to be a functional property as important as the foam-forming capacity. Emulsifying capacity achieved its highest value at pH 9, appearing similar to smooth butter. When proteins were exposed to pH 5, the isoelectric point, the emulsion did not form (Fig. 5). This suggests that protein–protein interactions were so strong that hydrophobic residues could not be exposed, thus avoiding an interaction between these residues and the oil phase to form the emulsion (Waniska *et al.* 1981; Jackman *et al.* 1989).

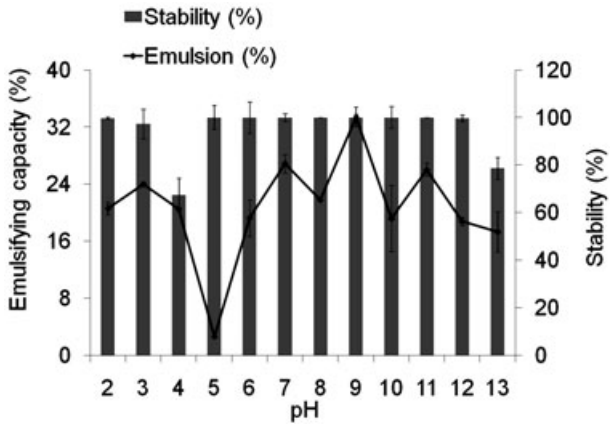

FIG. 5. EFFECT OF pH ON THE EMULSION-FORMING CAPACITY AND STABILITY OF JUMBO SQUID PROTEIN
Values represent the mean of at least three replicates; bars indicate SD.

Enzymatic Hydrolysis

The highest emulsifying capacity (32 ± 0.12%) was achieved with alcalase-produced hydrolysates prepared at pH 8 at 35C. The whippability of papain-produced hydrolysates was higher than that of alcalase-produced hydrolysates, with a maximum value of 245 ± 17.68% when the hydrolysates were prepared at pH 8 at 30C. Almost 80% of the protein could be recovered in a water-soluble form after hydrolysis with papain at pH 10 at the three temperatures (Table 1). However, from the hydrolysates produced by alcalase, less than 15% of the protein could be recovered under any operating condition. All results, including foam and emulsion stability, are shown in Table 1.

Enzymatic hydrolysis performed at moderate conditions of pH and temperature appears to be a suitable way to achieve solubilization of squid proteins and improve their functional properties. The use of papain provided 80% protein recovery in soluble form. Protein recovery and functional properties of jumbo squid proteins was not improved by the use of alcalase.

The highest value of whippability of squid protein was achieved when no enzyme was added at 50C and pH 8 (390.0 ± 0.1%) and foam stability was 100%. Under the same conditions (50C and pH 8), the highest value of emulsion-forming capacity (48.7 ± 3%) was achieved when no enzyme was added. However, its stability (~85%) was not so promising.

Improvement of the three functional properties of squid proteins is not the result of a single condition of temperature, pH or enzymatic hydrolysis. One could compromise the conditions that upgrade one property with the possibility of failure with the other two.

TABLE 1.
FUNCTIONAL PROPERTIES OF SQUID PROTEIN HYDROLYSATES

Enzyme	pH	T (C)	EC (%)	ES (%)	W (%)	FS (%)	PR (%)	DH (%)
Alcalase®	8	30	22.0 ± 0.1	35.0 ± 3.9	35.0 ± 10.0	75.3 ± 3.0	6.12 ± 0.12	8.32 ± 0.29
	8	35	32.0 ± 0.1	40.0 ± 0.0	40.0 ± 3.0	56.8 ± 3.3	8.53 ± 0.89	6.88 ± 0.57
	8	40	27.0 ± 0.1	24.2 ± 0.8	45.0 ± 5.0	89.8 ± 0.1	13.57 ± 0.07	4.14 ± 0.28
	9	30	21.1 ± 0.0	42.4 ± 1.1	41.5 ± 3.0	7.6 ± 3.9	3.8 ± 0.09	6.98 ± 0.44
	9	35	15.0 ± 0.0	75.5 ± 4.6	24.0 ± 6.0	48.9 ± 2.9	5.57 ± 0.93	8.56 ± 0.63
	9	40	3.4 ± 0.0	0.0 ± 0.0	2.5 ± 0.0	97.6 ± 0.0	11.56 ± 0.5	6.07 ± 0.37
	10	30	1.2 ± 0.2	0.0 ± 0.0	3.4 ± 1.0	87.5 ± 0.4	6.95 ± 0.53	11.1 ± 1.07
	10	35	1.0 ± 0.1	0.0 ± 0.0	2.4 ± 0.5	57.6 ± 0.2	9.52 ± 0.98	9.22 ± 0.94
	10	40	0.0 ± 0.0	0	0.0 ± 0.0	0.0	8.23 ± 0.1	8.11 ± 0.94
Papain	8	30	25.2 ± 0.9	102.7 ± 12.2	245.0 ± 17.7	100.0 ± 0.0	46.54 ± 0.62	4.85 ± 0.66
	8	35	24.6 ± 0.2	98.4 ± 9.4	175.0 ± 3.0	98.6 ± 0.6	53.91 ± 0.16	6.47 ± 0.85
	8	40	27.0 ± 1.0	100.0 ± 8.5	153.0 ± 2.0	95.4 ± 0.5	45.88 ± 1.1	8.01 ± 1.61
	9	30	22.8 ± 0.9	101.3 ± 3.5	156.3 ± 3.0	70.5 ± 4.9	21.97 ± 0.41	8.13 ± 0.55
	9	35	23.7 ± 1.9	103.1 ± 4.3	176.0 ± 0.0	100.0 ± 3.0	21.31 ± 1.66	4.45 ± 0.92
	9	40	21.7 ± 1.1	93.9 ± 0.6	137.0 ± 1.0	100.0 ± 1.7	19.58 ± 0.13	8.74 ± 0.77
	10	30	22.7 ± 1.4	94.4 ± 18.0	192.5 ± 1.4	69.6 ± 13.0	71.79 ± 4.9	5.24 ± 1.21
	10	35	20.7 ± 0.6	96.2 ± 9.7	157.0 ± 4.0	100.0 ± 0.0	76.75 ± 0.19	15.4 ± 1.21
	10	40	20.9 ± 0.3	101.2 ± 10.2	133.8 ± 1.8	52.4 ± 8.7	72.71 ± 1.29	11.2 ± 0.81
Endogenous	8	50	48.7 ± 3.0	84.7 ± 3.3	390.0 ± 0.1	100.0 ± 0.2	n.d.	n.d.

EC, emulsion forming capacity (%); ES, emulsion stability (%); W, whippability (%); FS, foam stability (%); PR, protein recovery (%); DH, degree of hydrolysis (%); n.d., not determined. Values are means of at least three replicates ± SD.

CONCLUSIONS

Jumbo squid is a rich source of protein. Squid proteins show high whippability, emulsifying capacity and particularly, solubility within a wide range of pH and temperature. These qualities could make jumbo squid proteins a useful and valuable ingredient in food products. The functional properties tested here suggest an effective way to take advantage of squid proteins and the possibility of adding value to this marine resource. However, more research is needed to improve the whole process, particularly the downstream part of the process.

Processing jumbo squid proteins must involve minimal exposure to temperatures higher than 45C, unless whippability needs to be improved. This functional property could become even better by increasing the pH, suggesting that squid proteins may be used as an ingredient in aerated products, such as meringues. The emulsifying capacity of jumbo squid proteins and their stability were improved at pH 9. Results suggest that jumbo squid protein could be recovered in a high-yield hydrolysis process with functional properties useful in different food product systems as an emulsifier or foam-producing ingredient.

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